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AGROINDUSTRIAL WASTE VALORISATION BY APPLICATION OF MEMBRANE TECHNOLOGY

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ABSTRACT

The oil seed industry is vast and worldwide growing agro industrial sector. Due to the rising world population, the consumption of refined vegetable oils has increased significantly, resulting in an increase in the production of seed by-products. The use of byproducts for valuable component production is a very good alternative for earning additional revenue and to solve related environmental disposal problems. The scope of this research work is to present the state-of-the-art for obtaining value added components (phytochemicals and nutraceuticals) from by-products of the vegetable oil industry emphasizing the expediency of membrane separation process. Aim of this study is to prepare valuable peptide components from agro industrial waste using ultrafiltration process. Use of ultrafiltration process in production of bioactive peptides in form of membrane bioreactors allows the continuous production of specific peptide sequences with functional and nutritional properties. Furthermore, recycling of the enzyme and elimination of residual proteins results in a much improved enzyme yield and process productivity level. Present research activities aims profitable utilization of agro-industrial waste employing the concept of membrane fractionation and membrane bioreactor emphasizing its potential in the field of food-biotechnology.

KEYWORDS: Oil seed, seed meal, agroindustrial waste, nutraceuticals, protein hydrolysate, food formulation.

INTRODUCTION:

Oil seeds are second largest grain crop in the supply of plant proteins for human and animal consumption. Oil seeds are rich in protein (30-45%) and defatted meal constitute a good source of protein, fiber and other micronutrients like polyphenol for human consumption. In Indian context, the utilization of oil seed meals in food and non food industries from sesame, mustard, sunflower are very much important because these are the major oil bearing materials in India. For utilization of these meals in food and pharmaceutical industries, proper technological development is required.

To meet the demand of the functional foods, a new generation of functional protein ingredients has to be created using innovative technology. One such product is protein isolate and respective protein hydrolysate. Modification of proteins by proteolytic enzymes is an effective way to improve the various functional properties and also to enhance utilization profile of proteins.[1-4] The peptides that are produced by partial hydrolysis of proteins have smaller molecular sizes and less secondary structure than the original proteins. In fact, the protein hydrolysate composed of short peptides with well-defined characteristics, are very much useful in producing the functional foods for specific physiological needs, such as malnutrition associated with cancer, burns, traumas and liver failure and for nutritional support of children with chronic or acute diarrhea or milk protein allergies. Protein hydrolysates can also be utilized as functional ingredients and flavor enhancers in foods, cosmetics, personal care products, confectionary and in the fortification of drinks and juices.[5, 6]. Protein hydrolysates are also used in many food systems to improve functional properties such as emulsifying, foaming and fat-water holding properties. Solubility of protein, emulsifying properties and foaming capacities can be improved with a limited degree of hydrolysis, whereas, excessive hydrolysis often cause loss of some of these functionalities.[7]

Small peptides (bioactive peptides) present in protein hydrolysate are mainly used in therapeutic applications like temporarily decrease of systolic blood pressure[8], in the treatment of psoriasis[9], age related various chronic diseases[10]. Synthetic small peptides containing three or six amino acid residues can improve the key parameters of monoclonal antibody- producing mouse hybridoma cultures.[11] The term bioactive peptides is used to identify molecules of peptidic nature or origin which display a biological behavior or activity which can be developed at the industrial level for pharmaceutical, diagnostic, chemical and agro-food applications. These bioactive peptides are small protein fragments produced by gastrointestinal digestion. The great advantage of using modified peptides instead of classical peptidomimetic is a very high bioavailability, a long lasting activity *in vivo*, and limited toxicity. Beside this, the costs for setting-up the synthetic strategy are quite low, when compared to those of setting-up stereo-specific synthetic routes.

Many efforts have been devoted in the last decade in the development of therapeutic agents based on peptides. The reason for this interest arises from the continuous discovery of new, highly-potent, biologically active peptides. So, it is possible to obtain modified peptides with superior properties for therapeutic applications. The major limitation in the use of peptides as drugs, such as bio-availability, life time, is in large extent related to the conformational flexibility of the bioactive peptides. So, considering these aspects it can be said that production of protein hydrolysate of improved functional properties from seeds are very much important from nutritional and commercial point of view.

The use of membrane technology (MT) in isolation and fractionation of seed protein has generated considerable research interest as evidenced by several recent papers[12-15] and patent applications. [16, 17] Being simple, selective and energy efficient, MT has significant advantages over other technologies. Additionally, membrane processes are compact, modular and easily amenable to automation and scale-up. Membranes have been found in large-volume and niche applications across a range of food and beverage industries, including fluid milk, cheese, and other dairy products, grain and oilseed products, beer, wine, and soft drinks, sugar and other sweeteners, meat, poultry, and seafood products, and various miscellaneous foods and food additives. Aim of this study is, to develop processes for synthesis of bioactive peptides from jatropha protein hydrolysate by using membrane process and their characterization for potential application in various food formulations.

MATERIALS AND METHODS:

Materials:

Jatropha oil seed were purchased from local market. Defatted Jatropha (*J. curcas L.*) seed cake was prepared in lab scale soxhlet apparatus using hexane as solvent. Phorbol-12-myristate 13-acetate was purchased from Sigma -Aldrich (USA). Folin-Ciocaltau's Phenol reagent (AR Grade, 2 N, Batch noX/729505) was supplied by SISCO research laboratory private limited, Mumbai, India. Papain (activity 6000NF unit batch no.2807 supplied by Viral Rasayan, West Bengal, India). PES membrane (5kDa MWCO, dia 76 mm) was purchased from Omega, Pall Life Sciences, USA. All other chemicals, except otherwise stated, were purchased from SRL (Sisco Research Laboratory, India) company limited. All results are expressed as average of three determinations.

Methods:

Analysis of chemical composition of *J. curcas* seed cake:

J. Curcas seed cake was ground and dried by a vacuum oven at 55±2°C until its weight was constant. It was analyzed for moisture, protein, fat, ash and fiber by the standard methods of the Association of Official Analytical Chemists (AOAC, 1995). Its carbohydrate content was calculated by difference method on dry basis. Phorbol esters were analysed following the procedures as described by Saetae et al., 2011.[18]

Detoxification study of *J. curcas* seed cake by bacterial fermentation:

In present case solid-substrate was employed for detoxification study (removal of phorbol ester from seed meal) following the procedure of Saetae et al., (2011)[18]. For solid-substrate fermentation, 1ml of freshly prepared bacterial cells were transferred to each of 250ml Erlenmeyer flasks containing 10 g sterile seed cake with an addition of sterile distilled water (pH 7.0± 0.1) to adjust the initial moisture content of the seed cake to 70±1%. The flasks were then hand shaken well and incubated at 30°C for 7 days. During incubation, the flasks were shaken periodically and samples were collected. At first day and at the end of fermentation, the flasks were analysed for PE content. Flasks containing seed cake without bacterial inoculation incubated under the same conditions as control experiments. The fermented seed cake samples were also dried by a vacuum oven at 55°C for 24 h. The dried sample obtained was used for the

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determination of phorbol esters.

Assessment of phorbol ester:
Phorbol esters were extracted from the seed cake following the method described by Saetae and Suntornsuk (2010).[19] The dry extract was dissolved by absolute methanol for phorbol ester determination by HPLC (CyberLab USA) at the wavelength of 280 nm. The analysis was carried out using a reversed phase chromatography C18 column (250 × 4 mm I.D and 5 µm particle size), gradient mobile phase comprising deionized water with 0.5% acetic acid (solvent A) and 100% acetonitrile (solvent B) in the ratio from 40:60 (v/v) until 100:0 (v/v) of A: B over 60 min at a flow rate of 1.3 mL/min and at room temperature. For preparation of standard curve Phorbol-12-myristate-13-acetate was used in the range of 0.005-0.5mg/ml.

Extraction and determination of protein content in meal:
Oil seed were collected from market and defatted seed flour is prepared in laboratory using food grade hexane in Soxhlet apparatus. The meal is milled and passed through 80-mesh screen (about 80% passed through) and mixed with water (1:10 w/v). The pH adjusted to 11 with 1 (N) NaOH solution, stirred for 1 h at 50- 55°C, and then centrifuged at 4000×g for 10 min (to remove the solid residual meal).The supernatant was separated and stored at 4°C for membrane processing to produce the protein isolate. Protein contents of the feed, retentate and permeate are determined according to Folin-Lowry method of protein assay at 750nm against appropriate blank.[20]

Determination of degree of hydrolysis:
Degree of hydrolysis, defined at the percentage of peptide bonds cleaved, was calculated by the determination of free amino groups by reaction with 2,4,6-trinitrobenzene sulphonic acid (TNBS) according to the method of Alder–Nissen.[21] 0.25 mL of a sample containing about 0.25×10^{-3} and 2.5×10^{-3} amino equiv/ L, is mixed in a test tube with 2.0mL of phosphate buffer at pH-8.2. 2 mL of TNBS solution was added and the test tube was shaken and placed in a water bath at 50°C for 60 min. During incubation, test tubes and the water bath maintained covered with aluminum foil because the blank reaction is accelerated by exposure to light. After 60 min 4.0 mL of 0.1 (N) HCL was added to terminate the reaction, and the test tube was allowed to stand at room temperature for 30 min before the absorbance was read against water at 340 nm. Total number of amino groups determined in a sample 100% hydrolysate at 110°C or 24 h in 6 (N) HCL (10 mg sample in 4 mL HCL).

Determination of emulsifying property:
Emulsification properties were measured by the method of Pearce and Kinsella. Pure soybean oil (2mL) and 6 mL of 0.1% protein solution (pH-8) were homogenized in a mechanical homogenizer at highest setting for 1 min. 50 mL portion of the emulsion were pipetted from the bottom of the container at 0 and 10 min after homogenization. Each portion was diluted with 5 mL of 0.1% SDS (sodium dodecyl sulphate) solution. Absorbance of these diluted solutions was measured at 500 nm with the aid of UV-VIS-spectrophotometer against the appropriate blank. The absorbance was measured immediately (Abs_0) and 10 min (Abs_{10}) after emulsion formation.

RESULTS AND DISCUSSION:

Proximate analysis of the Jatropha cake:
Sterilized *J. curcas* seed cake contained approximately 15% (w/w) protein, 2.9% (w/w) fat, 5.8% (w/w) ash, 7% (w/w) fiber and 65.5% (w/w) carbohydrate on dry basis (Table 1). Moisture content, fiber and fat content reduced to considerable extent after de-oiling and autoclave sterilisation, however carbohydrate and ash content increases significantly. In present study, the protein and fat content in seed and seed cake was found comparatively less which may be due to differences in seed diversity, and deoiling procedure as reported by several group of authors.[19,22-23]

Table 1: Proximate analysis of Jatropha seed and seed cake

Parameter	Seed	Seed cake
Moisture	4.75 ±0.35	3.0± 0.42
Ash	4.25±0.21	5.8±0.20
Lipid	32.75±0.20	2.9±0.10
Protein	20.10±0.12	19.1±0.24
Fiber	19.60±0.10	7.7±0.20
carbohydrate	18.55±0.10	61.5±0.10

Detoxification of japropha seed cake by *Bacillus mesentericus:*
As illustrated in Table 1, Jatropha is seed cake is rich in protein and carbohydrate and hence can be utilized as good substrate for growth of *Bacillus mesentericus*. Several publications have also reported the Jatropha cake as potential nutrient medium for microbial cultivation *A. niger, Penicillium chrysogenum, Rhizopus oligosporous, Rhizopus nigricans, Trichoderma longibrachitum*[23] *and Rhizopus oryzae*[24], *B. subtilis and B. licheniformis*[25].

In present case, *B. mesentericus* was observed well flourished on *J. curcas* seed cake in solid-substrate fermentation and about 68.33% growth in 7days. During the fermentation process pH of the medium was found increased to alkaline range (pH-8.7) possibly due to synthesis of protease which results amino acids and ammonia by protein digestion (24).

J. curcas seed cake contain phorbol esters about 2.6mg/g. After the sterilization process, considerable amount of phorbol esters retained in seed cake, being thermally stable.[25] Results showed that *B. mesentericus* have lowered phorbol esters content as illustrated in Table 2. It was also found that their reduction in the control experiments was negligible. The variation of phorbol ester content with time is illustrated in Figure 1. Concentrations effect of the microbial species have illustrated in Table 2. Overall results suggest that, about 97.2% removal of the PEs can be achieved by solid substrate fermentation process after 7 days incubation. The final concentration obtained during this study being close to the acceptable concentration region highlights the prospects of adopted technique in detoxifying the jatropha seed meal. Comparing the present experimental observation with the existing results outcomes[25], in can be unequivocally stated that this particular strain have immense potential to detoxify japropha seed cake and still not totally explored.

Table 2: Change in PE content after incubation

Conc. CFU/ml	PE content mg/g Seed cake	PE content mg/g Seed cake Sterilized	PE content mg/g Seed cake Fermented	Overall removal (%)
1×105	2.60±0.2	2.21±0.13	1.410±0.13	45.6±2.4
1×107	2.60±0.2	2.23±0.12	0.570±0.05	78.2±3.2
1×109	2.60±0.2	2.24±0.16	0.079±0.02	97.2±2.5

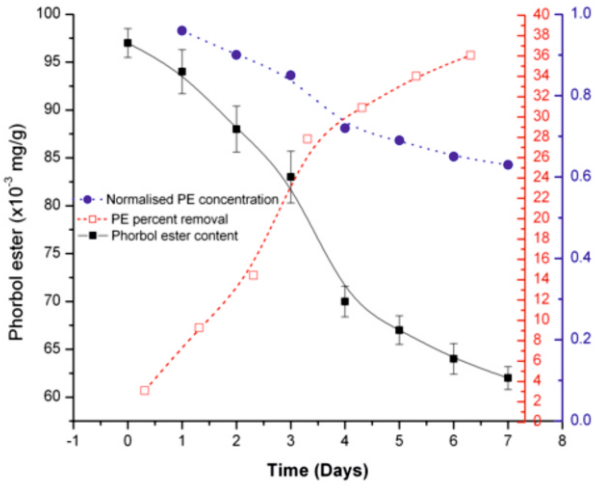

Figure 1: Removal of phorbol ester (PE) as function of time

Enzymatic hydrolysis and peptide yield value:
Enzymatic hydrolysis has been carried out with papain enzyme by varying the dose from 0.1% to 0.5%. Figure 2 is representing the degree of hydrolysis for protein isolate with changing dose of enzyme and time of hydrolysis. The degree of hydrolysis increases with the increasing papain concentration and with the time of hydrolysis. Upto 30 minute reaction time the degree of hydrolysis increases sharply with time for all doses of the enzyme, after that slop value changes gradually. Peptide yield value at different enzyme dosing value is presented in Table 3. Results shows significant increase in peptide yield value with increase in enzyme concentration.

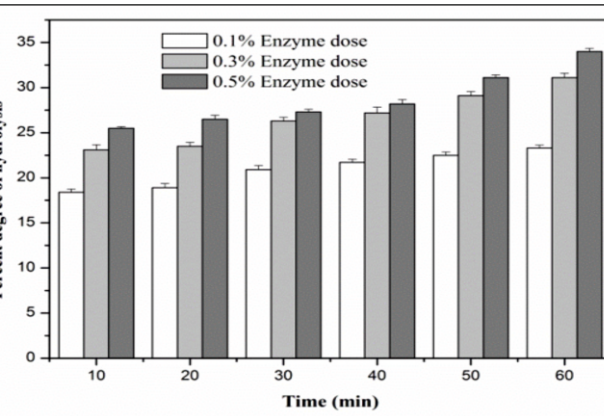

Figure 2: Effect of enzyme concentration and time on degree of hydrolysis of Jatropha protein isolate

Table 3: Peptide yield for hydrolysis time of 30 minutes with different enzyme dosing

Peptide yield	0.2%	0.5%
Molecular cut-off range 5000-2000Da (mg/100mg)	4.22±0.9	5.14±1.3

Table 4: Emulsifying properties of protein hydrolysates

Product Name	EAI (m^2/g) with different enzyme doses (w/w)		
	0.1%	0.3%	0.3%
PH 10	147.20±1.2	186.10±1.1	204.00±0.2
PH 20	151.20±1.0	186.90±1.2	187.60±0.8
PH 30	167.20±0.7	187.80±0.6	186.52±0.8
PH 40	173.60±0.9	186.30±0.8	185.00±0.8
PH 50	180.00±1.2	184.30±0.8	181.00±1.4
PH 60	186.40±1.4	181.00±1.1	179.00±1.6

Table 4 shows the emulsifying properties (emulsifying activity index, EAI) of different protein hydrolysates. The maximum EAI is obtained with 0.2% papain for 30 minute hydrolysis and 0.5% papain for 10 minute hydrolysis. At both these points degree of hydrolysis is about 25%. With further increasing degree of hydrolysis, no improvement of EAI is obtained. So, it can be said that maximum EAI corresponds to the degree of hydrolysis of 25%.

CONCLUSIONS:

Experimental results affirm that detoxification of *J. curcas* seed cake by *B. mesentericus* could remove the targeted toxin phorbol ester in the seed cake in significant amount. The detoxified seed meal after enzymatic hydrolysis in membrane bioreactor has shown improved functional properties and significant yield of particular peptide fractions. Hence fine tuning of the involved process can help in earning more incentive for agrochemical industries providing an alternative route of preparing functional food ingredient.

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